(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number
WO 01/61053 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/US01/05476

(22) International Filing Date: 20 February 2001 (20.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/506,178 17 February 2000 (17.02.2000) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/506,178 (CIP)
Filed on 17 February 2000 (17.02.2000)

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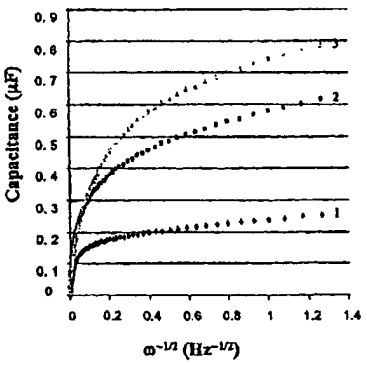
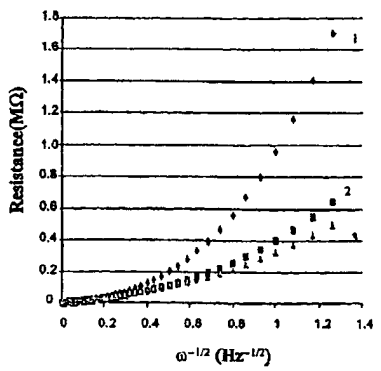
(54) Title: PROTEIN AND PEPTIDE SENSORS USING ELECTRICAL DETECTION METHODS

(57) Abstract: The present invention provides an apparatus and methods for the electrical detection of molecular interactions between a probe molecule and a protein or peptide target molecule, but without requiring the use of electrochemical or other reporters to obtain measurable signals. The methods can be used for electrical detection of molecular interactions between probe molecules bound to defined regions of an array and protein or peptide target molecules which are permitted to interact with the probe molecules.





WO 01/61053 A2

(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PROTEIN AND PEPTIDE SENSORS USING ELECTRICAL DETECTION METHODS

FIELD OF THE INVENTION

This invention relates to the electrical detection of molecular interaction between biological molecules. Specifically, the invention relates to electrical detection of interactions between a probe molecule and a target molecule, wherein the target molecule is a protein or peptide.

BACKGROUND OF THE INVENTION

A number of commonly-utilized biological applications rely on the ability of analytical technologies to readily detect events related to the interaction between probe and target molecules. However, these detection technologies have traditionally utilized radioactive isotopes or fluorescent compounds to monitor probe-target interactions. For example, Potyrailo et al., 1998, Anal. Chem. 70: 3419-25, describe an apparatus and method for detecting interactions between immobilized fluorescently-labeled aptamers and peptide target molecules. Furthermore, while immunoassays offer some of the most powerful techniques for the molecular detection of peptides, the most sensitive of these techniques requires the use of a fluorescently- or radioactively-labeled target or probe molecule.

Methods for the electrical or electrochemical detection of probe-target interactions have provided an attractive alternative to detection techniques relying on radioactive or fluorescent labels. Electrical or electrochemical detection techniques are based on the detection of alterations in the electrical properties of an electrode arising from interactions between one group of molecules attached to the surface of an electrode (often referred to as "probe" molecules) and another set of molecules present in a reaction mixture (often referred to as "target" molecules). Electrical or electrochemical detection eliminates many of the disadvantages inherent in use of radioactive or fluorescent labels to detect interactions between the probe and target molecules. This process offers, for example, a detection technique that is safe, inexpensive, and sensitive, and is not burdened with complex and onerous regulatory requirements.

However, despite these advantages, there are a number of obstacles in using electrical or electrochemical detection techniques for analyzing molecular interactions. One such obstacle is the requirement, in some methods, of incorporating an electrochemical label into

the target molecule. Labeled target molecules have been used to increase the electrical signal, thereby permitting molecular interactions between the target molecules and probe molecules to be more readily detected and at lower target concentrations. For example, Meade *et al.* (in U.S. Patent Nos. 5,591,578. 5,705,348. 5,770,369, 5,780,234 and 5,824,473) provide methods for the selective covalent modification of target molecules with redox-active moieties such as transition metal complexes. Meade *et al.* further disclose assays for detecting molecular interactions that employ such covalently-modified target molecules.

Certain alternative methods that do not employ labeled target molecules have been described in the prior art. For example, Hollis *et al.* (in U.S. Patent Nos. 5,653,939 and 5,846,708) provide a method and apparatus for identifying molecular structures within a sample substance using a monolithic array of test sites formed on a substrate upon which the sample substance is applied. In the method of Hollis *et al.*, changes in the electromagnetic or acoustic properties—for example, the change in resonant frequency—of the test sites following the addition of the sample substance are detected in order to determine which probes have interacted with target molecules in the sample substance.

In addition, Eggers *et al.* (in U.S. Patent Nos. 5,532,128, 5,670,322, and 5,891.630) provide a method and apparatus for identifying molecular structures within a sample substance. In the method of Eggers *et al.* a plurality of test sites to which probes have been bound is exposed to a sample substance and then an electrical signal is applied to the test sites. Changes in the dielectrical properties of the test sites are subsequently detected to determine which probes have interacted with target molecules in the sample substance.

Another obstacle in the development of a simple and cost-effective electrical and electrochemical detection apparatus for detecting molecular interactions involves limitations in how probe molecules have been attached to electrodes. This is particularly important in fabricating arrays of probes, such as microarrays known in the art. For example, the prior art provides microarrays using polyacrylamide pads for attachment of oligonucleotide probes to a solid support. However, the art has not provided such pads in conjunction with electrodes in an electrical or electrochemical detection apparatus.

Yang *et al.*, 1997, Anal. Chim. Acta 346: 259-75 describe fabrication of microarrays having immobilized probe molecules wherein molecular interactions between labeled target molecules and probes that have been directly attached to solid electrodes are detected using

electrical or electrochemical means. Yang *et al.*, however do not suggest using electrical or electrochemical detection techniques in combination with the immobilization of probes on polyacrylamide gel pads.

There remains a need in the art to develop alternatives to current detection methods used to detect interactions between biological molecules, particularly molecular interactions involving protein or peptide target molecules. More particularly, there is a need in the art to develop electrical detection methods for detecting interactions between biological molecules that do not require modifying target or probe molecules with reporter labels. The development of such methods would have wide application in the medical, genetic, and molecular biological arts. There further remains a need in the art to develop alternative methods for attaching biological probe molecules to the microelectrodes of an electrical or electrochemical device. Thus, there remains a need in the art to develop inexpensive and safe alternatives to standard immunological and molecular detection methods.

SUMMARY OF THE INVENTION

The present invention provides an apparatus and methods for the electrical detection of molecular interactions between a probe molecule and a protein or peptide target molecule, but without requiring the use of electrochemical or other reporters to obtain measurable signals. The methods can be used for electrical detection of molecular interactions between probe molecules bound to defined regions of an array and protein or peptide target molecules that interact with the probe molecules.

The apparatus of the present invention comprises a supporting substrate, one or a plurality of microelectrodes in contact with the supporting substrate, one or a plurality of linking moieties in contact with the microelectrodes and to which probe molecules are immobilized, at least one counter-electrode in electrochemical contact with the microelectrodes, a means for producing an electrical signal at each microelectrode, a means for detecting changes in the electrical signal at each microelectrode and an electrolyte solution in contact with the one or a plurality of microelectrodes, linking moieties, and counter-electrodes.

The apparatus of the present invention may advantageously further comprise at least one reference electrode. The apparatus may also further comprise a plurality of wells, each of

which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode that is sufficient to interrogate the microelectrodes in contact with linker moieties.

The apparatus and methods of the present invention are useful for the electrical detection of molecular interactions between probe molecules immobilized on linker moieties in contact with microelectrodes and protein or peptide target molecules in a sample solution. In methods of the present invention, a first electrical signal is detected in a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized, the plurality of microelectrodes in contact with linker moieties is exposed to a sample mixture containing protein or peptide target molecules, and a second electrical signal is detected at the plurality of microelectrodes in contact with linker moieties. The first and second electrical signals are then compared, and molecular interactions between immobilized probe molecules and protein or peptide target molecules in the sample mixture are detected by determining that and by how much the first electrical signal is different from the second electrical signal.

The present invention provides an apparatus and methods for the electrical detection of molecular interactions between probe molecules and protein or peptide target molecules, but without the requirement that electrochemical reporters or labeled target molecules be used. As a result, when compared with methods disclosed in the prior art, the apparatus and methods of the present invention are capable of detecting molecular interactions between probe molecules and protein or peptide target molecules that are safer, less expensive, simpler, and that have an increased reproducibility and sensitivity compared to prior art methods.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate a schematic representation of the structure of a microelectrode in contact with a polyacrylamide gel linker moiety (*i.e.*, a porous hydrogel microelectrode) (Figure 1A) and a schematic representation of the structure of the tip of the porous hydrogel microelectrode (Figure 1B);

Figure 2 illustrates a porous hydrogel microelectrode;

Figures 3A and 3B illustrate plots of capacitance versus frequency (Figure 3A) and resistance versus frequency (Figure 3B) for a streptavidin-modified porous hydrogel microelectrode before (curve 1) and after immobilization of rabbit anti-BAP antibody (curve 2), and following incubation with BAP antigen (curve 3); and

Figures 4A and 4B illustrate plots of capacitance versus frequency (Figure 4A) and resistance versus frequency (Figure 4B) for a streptavidin-modified porous hydrogel microelectrode with immobilized rabbit anti-BAP antibody before (curve 1) and after incubation with BAP antigen (curve 2), and following incubation with an anti-rabbit IgG secondary antibody (curve 3).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The apparatus and methods of the present invention are useful for the electrical detection of molecular interactions between probe molecules immobilized by linker moieties in contact with microelectrodes and protein or peptide target molecules in a sample mixture.

As used herein, the term "array" refers to an ordered spatial arrangement, particularly an arrangement of immobilized biomolecules, such as specific binding molecules or probes as further described below. The present system finds particular utility in array formats, *i.e.* wherein there is a matrix of addressable locations (herein generally referred to as "pads", "addresses" or "micro-locations"). "Array" or grammatical equivalents thereof as used herein mean a plurality of probes in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different probes to many thousands can be made. Generally, the array will comprise from two to as many as 100,000 or more per cm^2, depending on the size of the pads, as well as the end use of the array. Preferred ranges are from about 2 to about 10,000, with from about 5 to about 1000 being preferred, and from about 10 to about 100 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single probe may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

As used herein, the terms "bioarray," "biochip" and "biochip array" refer to an ordered spatial arrangement of immobilized biomolecules on a microelectrode arrayed on a solid supporting substrate. Preferred probe molecules include nucleic acids, oligonucleotides, peptides, ligands, antibodies and antigens; peptides and proteins are the most preferred probe species. Biochips, as used in the art, encompass substrates containing arrays or microarrays, preferably ordered arrays and most preferably ordered, addressable arrays, of biological molecules that comprise one member of a biological binding pair. Typically, such arrays are oligonucleotide arrays comprising a nucleotide sequence that is complementary to at least one sequence that may be or is expected to be present in a biological sample. Alternatively, and preferably, proteins, peptides or other small molecules can be arrayed on such biochips for performing, *inter alia*, immunological analyses (wherein the arrayed molecules are antigens), assaying biological receptors (wherein the arrayed molecules are ligands, agonists or antagonists of said receptors). In alternative embodiments, the probes of the invention are specific for a particular species, subspecies or strain of bacteria, and most preferably are specific for viable bacteria of said species, subspecies or strain.

As used herein, the term "addressable array" refers to an array wherein the individual elements have precisely defined x and y coordinates, so that a given element at a particular position in the array can be identified.

As used herein, the terms "probe", "biomolecular probe", binding molecules", "binding ligand", or grammatical equivalents thereof refer to a molecule (preferably a biomolecule) used to detect another biomolecule. Examples include antigens that detect antibodies, oligonucleotides that detect complimentary oligonucleotides, and ligands that detect receptors. Such probes are preferably immobilized on a microelectrode comprising a substrate.

"Binding ligand" or grammatical equivalents thereof as used herein means a compound that is used to probe for the presence of the target analyte, and that will bind to the target analyte. As will be appreciated by those in the art, the composition of the binding ligand will depend on the composition of the target analyte. Binding ligands for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the binding ligands include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules. When the analyte is a metal

ion, the binding ligand generally comprises traditional metal ion ligands or chelators. Preferred binding ligand proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. The binding ligand may be nucleic acid, when nucleic acid binding proteins are the targets; alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents (hereby incorporated by reference) nucleic acid "aptomers" can be developed for binding to virtually any target analyte. Similarly, there is a wide body of literature relating to the development of binding partners based on combinatorial chemistry methods. In this embodiment, when the binding ligand is a nucleic acid, preferred compositions and techniques are outlined in PCT US97/20014, hereby incorporated by reference.

As used herein "nucleic acid", "oligonucleotide"or grammatical equivalents mean at least two nucleotides covalently linked together. A nucleic acid according to the present invention will generally contain phosphodiester bonds, although in some cases nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage *et al.*, Tetrahedron 49(10):1925 (1993); Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl *et al.*, Eur. J. Biochem. 81:579 (1977); Letsinger *et al.*, Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger *et al.*, J. Am. Chem. Soc. 110:4470 (1988); and Pauwels *et al.*, Chemica Scripta 26:141 (1986)), phosphorothioate (Mag *et al.*, Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu *et al.*, J. Am. Chem. Soc. 111:2321 (1989)), O-methylphophoroamidite linkages (*see* Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (*see* Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier *et al.*, Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson *et al.*, Nature 380:207 (1996)) (all of these references are incorporated herein in their entirety by reference). Other analog nucleic acids include those with positive backbones (Denpcy *et al.*, Proc. Natl. Acad. Sci. USA 92:6097 (1995)), non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi *et al.*, Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger *et al.*, J. Am. Chem. Soc. 110:4470 (1988); Letsinger *et al.*, Nucleoside & Nucleotide

13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker *et al.*, Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs *et al.*, J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)), and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (*see* Jenkins *et al.*, Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention.

In a preferred embodiment, the binding of the target analyte to the binding ligand is specific, and the binding ligand is part of a binding pair. As used herein "specifically bind" means that the ligand binds the analyte with specificity sufficient to differentiate between the analyte and other components or contaminants within the test sample. However, as will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the disassociation constants of the analyte to the binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

In a preferred embodiment probes or targets may be proteins. As used herein "peptides", "polypeptides", "proteins" or grammatical equivalents thereof mean proteins, oligopeptides, peptides, derivatives and analogs thereof (including without limitation proteins containing non-naturally occurring amino acids and amino acid analogs), and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration.

In a preferred embodiment, the amino acids are in the (S) or L-configuration. When the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

In some embodiments of the present invention, the probe molecules of the apparatus comprise proteins or peptides. The protein or peptide probe molecules of the present invention are preferably peptides comprising from about 5 to about 100 amino acids, more preferably antigen-recognizing peptieds or polypeptides belonging to the immunoglobulin superfamily. Said peptide or polypeptide probe molecules are immobilized to the microelectrodes of the invention through linker moieties, using techniques known to those with skill in the art wherein said linkage does not interfere with or inhibit the ability of the probe molecules to interact with protein or peptide target molecules in the sample mixture. As used herein "immobilize" or grammatical equivalents thereof means that the probe molecule is fixed relative to the microelectrode. For example and without limitation a probe molecule may be attached to a linker moiety, the probe molecule may be embedded within a matrix of a linker moiety, or any combination thereof, and the linker moiety is attached to the microelectrode. It will be appreciated that direct attachment of the probe molecule to the electrode is possible; howerver, this is not preferred. Attachment of the probe to a linker moiety is well within the skill of the artisan, and may include without limitation covalent bonding, ionic bonding, van der Waals forces, hydro-phobic/philic interactions, biologic attachment (such as avidin-streptavidin interactions), the latter being preferred.

In one preferred embodiment antibodies are used as probes. A protein may be used to generate polyclonal and monoclonal antibodies to proteins, which are useful as described herein.

In a preferred embodiment, the epitope of the probe is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity to other proteins. As used herein "epitope", "determinant", or grammatical equivalents thereof means a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Apop3 protein will be able to bind to the full length protein. The term "antibody" includes antibody fragments, as are known in the art, including Fab, Fab_2, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA

technologies. The term "antibody" further comprises polyclonal antibodies and monoclonal antibodies, which can be agonist or antagonist antibodies.

The proteins, whether a target or a probe, may be from any organism, including prokaryotes and eukaryotes, with enzymes from bacteria, fungi, extremeophiles such as the archebacteria, insects, fish, animals (particularly mammals and particularly human) and birds all possible. Specifically included within the definition of "protein" are fragments and domains of known proteins, including functional domains such as enzymatic domains, binding domains, etc., and smaller fragments, such as turns, loops, etc. That is, portions of proteins may be used as well. In addition, "protein" as used herein includes proteins, oligopeptides and peptides. In addition, protein variants, *i.e.* non-naturally occuring protein analog structures, may be used.

Suitable proteins for probes or targets include, but are not limited to, industrial and pharmaceutical proteins, including ligands, cell surface receptors, antigens, antibodies, cytokines, hormones, transcription factors, signaling modules, cytoskeletal proteins and enzymes. Suitable classes of enzymes include, but are not limited to, hydrolases such as proteases, carbohydrases, lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases, oxidoreductases, and phophatases. Suitable enzymes are listed in the Swiss_Prot enzyme database. Suitable protein backbones include, but are not limited to, all of those found in the protein data base compiled and serviced by the Research Collaboratory for Structural Bioinformatics (RCSB, formerly the Brookhaven National Lab).

Specifically, preferred target proteins include, but are not limited to, those with known structures (including variants) including cytokines (IL-1ra (+receptor complex), IL-1 (receptor alone), IL-1a, IL-1b (including variants and or receptor complex), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-β, INF-γ, IFN-α-2a; IFN-α-2B, TNF-α; CD40 ligand (chk), Human Obesity Protein Leptin, Granulocyte Colony-Stimulating Factor, Bone Morphogenetic Protein-7, Ciliary Neurotrophic Factor, Granulocyte-Macrophage Colony-Stimulating Factor, Monocyte Chemoattractant Protein 1, Macrophage Migration Inhibitory Factor, Human Glycosylation-Inhibiting Factor, Human Rantes, Human Macrophage Inflammatory Protein 1 Beta, human growth hormone, Leukemia Inhibitory Factor, Human Melanoma Growth Stimulatory Activity, neutrophil activating peptide-2, Cc-Chemokine Mcp-3, Platelet Factor M2, Neutrophil Activating Peptide 2, Eotaxin, Stromal Cell-Derived

Factor-1, Insulin, Insulin-like Growth Factor I, Insulin-like Growth Factor II, Transforming Growth Factor B1, Transforming Growth Factor B2, Transforming Growth Factor B3, Transforming Growth Factor A, Vascular Endothelial growth factor (VEGF), acidic Fibroblast growth factor, basic Fibroblast growth factor, Endothelial growth factor, Nerve growth factor, Brain Derived Neurotrophic Factor, Ciliary Neurotrophic Factor, Platelet Derived Growth Factor, Human Hepatocyte Growth Factor, Glial Cell-Derived Neurotrophic Factor, (as well as the 55 cytokines in PDB 1/12/99)); Erythropoietin; other extracellular signalling moeities, including, but not limited to, hedgehog Sonic, hedgehog Desert, hedgehog Indian, hCG; coaguation factors including, but not limited to, TPA and Factor VIIa; transcription factors, including but not limited to, p53, p53 tetramerization domain, Zn fingers (of which more than 12 have structures), homeodomains (of which 8 have structures), leucine zippers (of which 4 have structures); antibodies, including, but not limited to, cFv; viral proteins, including, but not limited to, hemagglutinin trimerization domain and hiv Gp41 ectodomain (fusion domain); intracellular signalling modules, including, but not limited to, SH2 domains (of which 8 structures are known), SH3 domains (of which 11 have structures), and Pleckstin Homology Domains; receptors, including, but not limited to, the extracellular Region Of Human Tissue Factor Cytokine-Binding Region Of Gp130, G-CSF receptor, erythropoietin receptor, Fibroblast Growth Factor receptor, TNF receptor, IL-1 receptor, IL-1 receptor/IL1ra complex, IL-4 receptor, INF-γ receptor alpha chain, MHC Class I, MHC Class II , T Cell Receptor, Insulin receptor, insulin receptor tyrosine kinase and human growth hormone receptor. It will be understood fragments and domains of these proteins, including functional domains, may also be used as probes.

In an alternative embodiment, the target molecules are proteins as defined above. In a preferred embodiment, the target molecules are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eukaryotic proteins may be made for screening in accordance with the present invention. Particularly preferred in this embodiment are bacterial, fungal, viral, and mammalian proteins (or libraries thereof), with the latter being preferred, and human proteins being especially preferred.

As will be appreciated, the probe or target molecules may comprise a natural products library, a peptide library, a phage display library, or a combinatorial library.

Suitable target protein molecules include, but are not limited to, (1) immunoglobulins, particularly IgEs, IgGs and IgMs, and particularly therapeutically or diagnostically relevant antibodies, including but not limited to, for example, antibodies to human albumin, apolipoproteins (including apolipoprotein E), human chorionic gonadotropin, cortisol, α-fetoprotein, thyroxin, thyroid stimulating hormone (TSH), antithrombin, antibodies to pharmaceuticals (including antieptileptic drugs (such as phenytoin, primidone, carbariezepin, ethosuximide, valproic acid, and phenobarbitol), cardioactive drugs (such as digoxin, lidocaine, procainamide, and disopyramide), bronchodilators (such as theophylline), antibiotics (such as chloramphenicol, sulfonamides), antidepressants, immunosuppresants, abused drugs (such as amphetamine, methamphetamine, cannabinoids, cocaine and opiates), and antibodies to any number of viruses (such as orthomyxoviruses, (*e.g.*, influenza virus), paramyxoviruses (*e.g.*, respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (*e.g.*, rubella virus), parvoviruses, poxviruses (*e.g.*, variola virus, vaccinia virus), enteroviruses (*e.g.*, poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (*e.g.*, Herpes simplex virus, varicella_zoster virus, cytomegalovirus, Epstein_Barr virus), rotaviruses, Norwalk viruses, hantavirus, arenavirus, rhabdovirus (*e.g.*, rabies virus), retroviruses (including HIV, HTLV_I and _II), papovaviruses (*e.g.*, papillomavirus), polyomaviruses, and picornaviruses.), and bacteria (including a wide variety of pathogenic and non_pathogenic prokaryotes of interest including Bacillus; Vibrio, *e.g.*, *V. cholerae;* Escherichia, *e.g.*, Enterotoxigenic *E. coli*, Shigella, *e.g.*, *S. dysenteriae;* Salmonella, *e.g.*, *S. typhi;* Mycobacterium *e.g.*, *M. tuberculosis, M. leprae*; Clostridium, *e.g.*, *C. botulinum, C. tetani, C. difficile, C.perfringens*; Cornyebacterium, *e.g.*, *C. diphtheriae*; Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g.*, *S. aureus*; Haemophilus, *e.g.*, *H. influenzae*; Neisseria, *e.g.*, *N. meningitidis, N. gonorrhoeae*; Yersinia, *e.g.*, *G. lambliaY. pestis*, Pseudomonas, *e.g.*, *P. aeruginosa, P. putida*; Chlamydia, *e.g.*, *C. trachomatis*; Bordetella, *e.g.*, *B. pertussis*; Treponema, *e.g.*, *T. palladium;* and the like); (2) enzymes (and other proteins), including but not limited to, enzymes used as indicators of or treatment for heart disease, including creatine kinase, lactate dehydrogenase, aspartate amino transferase, troponin T, myoglobin,

fibrinogen, cholesterol, triglycerides, thrombin, tissue plasminogen activator (tPA); pancreatic disease indicators including amylase, lipase, chymotrypsin and trypsin; liver function enzymes and proteins including cholinesterase, bilirubin, and alkaline phosphotase; aldolase, prostatic acid phosphatase, terminal deoxynucleotidyl transferase, and bacterial and viral enzymes such as HIV protease; (3) hormones and cytokines (many of which serve as ligands for cellular receptors) such as erythropoietin (EPO), thrombopoietin (TPO), the interleukins (including IL-1 through IL-17), insulin, insulin-like growth factors (including IGF-1 and -2), epidermal growth factor (EGF), transforming growth factors (including TGF-α and TGF-β), human growth hormone, transferrin, epidermal growth factor (EGF), low density lipoprotein, high density lipoprotein, leptin, VEGF, PDGF, ciliary neurotrophic factor, prolactin, adrenocorticotropic hormone (ACTH), calcitonin, human chorionic gonadotropin, cotrisol, estradiol, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), leutinzing hormone (LH), progeterone, testosterone; and (4) other proteins (including α-fetoprotein, carcinoembryonic antigen CEA.

In addition, any of the biomolecules for which antibodies are tested may be tested directly as well; that is, the virus or bacterial cells, therapeutic and abused drugs, etc., may be the test molecules.

In preferred embodiments, the apparatus of the present invention comprises a supporting substrate, one or a plurality of microelectrodes in contact with the supporting substrate, one or a plurality of linking moieties in contact with the microelectrodes and to which specific binding molecules are immobilized, at least one counter-electrode in electrochemical contact with the microelectrodes, a means for producing an electrical signal at each microelectrode, a means for detecting changes in the electrical signal at each microelectrode, and an electrolyte solution in contact with the microelectrodes, linking moieties, and counter-electrode.

In some embodiments of the present invention, the specific binding molecules of the apparatus comprise proteins or peptides. In one preferred embodiment, the specific binding molecules are antibodies. The antibodies immobilized on the linker moieties of the apparatus of the invention may be polyclonal or monoclonal antibodies, F(ab) fragments, F(ab)' fragments, $F(ab)_2$ fragments, or F_v fragments of polyclonal or monoclonal antibodies, or F(ab) or single chain antibodies selected from *in vitro* libraries. In alternative embodiments of the

present invention, the specific binding molecules are nucleic acids, oligonucleotides, or combinations thereof. In one preferred embodiment of the present invention, the specific binding molecules are aptamers (*i.e.*, oligonucleotides capable of interacting with target molecules such as peptides). Natural products libraries (such as, *inter alia*, yeast extracts), phage display libraries, or combinatorial libraries may also be used as specific binding molecules.

In one preferred embodiment of the present invention, the specific binding molecules are polyclonal antibodies and the antibodies are immobilized on the linker moieties using any technique known in the art that does not interfere with or inhibit the ability of the antibodies to specifically bind to a target analyte, such as and without limitation the conjugate antigen or portion thereof. In preferred embodiments, polyclonal antibodies, antisera, monoclonal antibodies, fragments of said polyclonal or monoclonal antibodies, or other specific ligand binding molecules, are immobilized onto and attached to the linker moieties of the apparatus of the invention using biotinylated species thereof that are conjugated with streptavidin added to the linker moiety material. In more preferred embodiments, the linker moiety material to which the streptavidin is added is polyacrylamide gel. In other embodiments the biotinylated specific binding molecule is a protein, peptide, nucleic acid, or oligonucleotide. Alternatively and as further described below, the specific binding molecules are embedded within a matrix, *e.g.*, polymer matrix, of the linker molecules.

In some embodiments of the present invention, the linker moieties of the apparatus are composed of materials including, but not limited to, polyacrylamide gel, agarose gel, polyethylene glycol, cellulose gel, or sol gels. In preferred embodiments, the linker moieties comprise polyacrylamide gel, which form a gel pad. In alternative embodiments of the present invention, the linker moieties, or gel pad comprise a conjugated polymer or copolymer film. Such conjugated polymer or copolymer film is composed of materials including, but not limited to, polypyrrole, polythiphene, polyaniline, polyfuran, polypyridine, polycarbazole, polyphenylene, poly(phenylenvinylene), polyfluorene, or polyindole, or their derivatives, their copolymers, and combinations thereof. In preferred embodiments, the linker moieties comprise a neutral pyrrole matrix, and the probes are embedded therein.

Polymeric hydrogels and gel pads are used as binding layers to adhere biological molecules to substrate surfaces, which biomolecules include, but are not limited to, proteins,

peptides, oligonucleotides, polynucleotides, and larger nucleic acid fragments. The oligonucleotide probes may be bound to the surface of a continuous layer of the hydrogel, to an array of gel pads, or embedded therein. The gel pads comprising biochips for use with the apparatus of the present invention are conveniently produced as thin sheets or slabs, typically by depositing a solution of acrylamide monomer, a crosslinker such methylene bisacrylamide, and a catalyst such as N, N, N', N' - tetramethylethylendiamine (TEMED) and an initiator such as ammonium persulfate for chemical polymerization, or 2,2-dimethoxy-2-phenyl-acetophone (DMPAP) for photopolymerization, in between two glass surfaces (e.g., glass plates or microscope slides) using a spacer to obtain the desired thickness of the polymeric gel. Generally, the acrylamide monomer and crosslinker are prepared in one solution of about 4-5% acrylamide (having an acrylamide/ bisacrylamide ratio of 19/1) in water/glycerol, with a nominal amount of initiator added. The solution is polymerized and crosslinked either by ultraviolet (UV) radiation (*e.g.*, 254nm for at least about 15 minutes, or other appropriate UV conditions, collectively termed "photopolymerization"), or by thermal initiation at elevated temperature (e.g., typically at about 40° C). Following polymerization and crosslinking, the top glass slide is removed from the surface to uncover the gel. The pore size (and hence the "sieving properties") of the gel is controlled by varying the amount of crosslinker and the percent solids in the monomer solution. The pore size also can be controlled by varying the polymerization temperature.

In the fabrication of arrays of polyacrylamide (i.e., patterned gels), in an embodiment of the present invention, the acrylamide solution typically is imaged through a mask during the UV polymerization/crosslinking step. The top glass slide is removed after polymerization, and the unpolymerized monomer is washed away (developed) with water, leaving a fine feature pattern of polyacrylamide hydrogel, which is used to produce the crosslinked polyacrylamide hydrogel pads. Further, in an application of lithographic techniques known in the semiconductor industry, light can be applied to discrete locations on the surface of a polyacrylamide hydrogel to activate these specified regions for the attachment of an oligonucleotide, an antibody, an antigen, a hormone, hormone receptor, a ligand or a polysaccharide on the surface (e.g., a polyacrylamide hydrogel surface) of a solid substrate (*see, e.g,* WO 91/07087, incorporated herein by reference).

For hydrogel-based arrays using polyacrylamide, biomolecules (such as oligonucleotides, peptides, polypeptides, or proteins) are covalently attached by forming an amide, ester or disulfide bond between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of the biomolecule to the polymer is usually performed after polymerization and chemical cross-linking of the polymer is completed.

Alternatively, oligonucleotides bearing 5'-terminal acrylamide modifications can be used that efficiently copolymerize with acrylamide monomers to form DNA-containing polyacrylamide copolymers (Rehman *et al.*, 1999, *Nucleic Acids Research* 27: 649-655). Using this approach, stable probe-containing layers can be fabricated on substrates (*e.g.*, microtiter plates and silanized glass) having exposed acrylic groups. This approach has made available the commercially marketed "Acrydite™" capture probes (available from Mosaic Technologies, Boston, MA). The Acrydite moiety is a phosporamidite that contains an ethylene group capable of free-radical copolymerization with acrylamide, and which can be used in standard DNA synthesizers to introduce copolymerizable groups at the 5' terminus of any oligonucleotide probe.

Alternatively, the linker moieties or gel pads may be formed using electrochemical techniques. For example, cyclic voltammetry of pyrrole, 3-acetateN--hydrodysuaccinimido pyrrole and PBS buffer, in the presence of probe molecules, forms a polypyrrole/probe film on a microelectrode surface. The probe molecules are embedded or fixed within the film. Other methods of embedding probe molecules within a gel pad matrix will be known to the skilled artisan. For example, polymerization of polyacrylamide gel in the presence of the probe molecules will embed the probe molecules therein.

The solid substrate can be made of a wide variety of materials and can be configured in a large number of ways, as is discussed herein and will be apparent to one of skill in the art. In addition, a single device may comprise more than one substrate; for example, there may be a "sample treatment" cassette that interfaces with a separate "detection" cassette; a raw sample is added to the sample treatment cassette and is manipulated to prepare the sample for detection, which is removed from the sample treatment cassette and added to the detection cassette. There may be an additional functional cassette into which the device fits; for example, a heating element which is placed in contact with the sample cassette to effect

reactions such as PCR. In some cases, a portion of the substrate may be removable; for example, the sample cassette may have a detachable detection cassette, such that the entire sample cassette is not contacted with the detection apparatus. See for example U.S. Patent No. 5,603,351, PCT US96/17116, and "Multilayered Microfluidic Devices for Analyte Reactions" filed in the PCT December 11, 2000, Serial No. PCT/US00/33499, hereby incorporated by reference.

The composition of the solid substrate will depend on a variety of factors, including the techniques used to create the device, the use of the device, the composition of the sample, the analyte to be detected, the size of the wells and microchannels, the presence or absence of electronic components, etc. Generally, the devices of the invention should be easily sterilizable as well.

The supporting substrate of the apparatus of the invention is advantageously made from any solid material, including but not limited to silicon such as silicon wafers, silicon dioxide, silicon nitride, glass and fused silica, gallium arsenide, indium phosphide, aluminum, ceramics, polyimide, quartz, plastics, resins and polymers including polymethylmethacrylate, acrylics, polyethylene, polyethylene terepthalate, polycarbonate, polystyrene and other styrene copolymers, polypropylene, polytetrafluoroethylene, superalloys, zircaloy, steel, gold, silver, copper, tungsten, molybdeumn, tantalum, Kovar™, Kevlar™, Kapton™, Mylar™, brass, sapphire, etc. High melting borosilicate or fused silicas may be preferred for their UV transmission properties when any of the sample manipulation steps require light based technologies. In addition, as outlined herein, portions of the internal surfaces of the device may be coated with a variety of coatings as needed, to reduce non-specific binding, to allow the attachment of binding ligands, for biocompatibility, for flow resistance, etc. In preferred embodiments, the supporting substrate of the apparatus of the present invention is composed of silicon or glass. The linker moieties are embedded within or placed in contact with the supporting substrate.

In another preferred embodiment, the substrate is made from printed circuit board (PCP) materials, including without limitation, fiberglass, teflon, ceramics, glass, silicon, mica, plastic (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polycarbonate, polyurethanes, Teflon™, and derivatives thereof, etc.), GETEK (a blend of polypropylene oxide and fiberglass), etc.

The supporting substrate has a surface area of from about 0.01μm^2 to about 5 cm^2 containing between 1 and 1 x 10^8 microelectrodes in contact with said linker moieties. In a preferred embodiment, the supporting substrate has a surface area of 10,000 μm^2 and contains 10^4 microelectrodes in contact with linker moieties. In preferred embodiments, the microelectrodes are arranged on the supporting substrate so that they are separated by a distance of from about 0.05μm to 0.5mm. In more preferred embodiments, the microelectrodes are regularly spaced on the solid supporting substrate with a uniform spacing there between.

In general, circuit board materials are those that comprise an insulating substrate that is coated with a conducting layer and processed using lithography techniques, particularly photolithography techniques, to form the patterns of electrodes and interconnects (sometimes referred to in the art as interconnections or leads). The insulating substrate is generally, but not always, a polymer. As is known in the art, one or a plurality of layers may be used, to make either "two dimensional" (e.g. all electrodes and interconnections in a plane) or "three dimensional" (wherein the electrodes are on one surface and the interconnects may go through the board to the other side) boards. Three dimensional systems frequently rely on the use of drilling or etching, followed by electroplating with a metal such as copper, such that the "through board" interconnections are made. Circuit board materials are often provided with a foil already attached to the substrate, such as a copper foil, with additional copper added as needed (for example for interconnections), for example by electroplating. The copper surface may then need to be roughened, for example through etching, to allow attachment of the adhesion layer.

In an alternative embodiment the solid substrate comprises ceramic materials, such as are outlined in U.S.S.N.s 09/235,081; 09/337,086; 09/464,490; 09/492,013; 09/466,325; 09/460,281; 09/460,283; 09/387,691; 09/438,600; 09/506,178; and 09/458,534; all of which are expressly incorporated by reference in their entirety. In this embodiment, the devices are made from layers of green-sheet that have been laminated and sintered together to form a substantially monolithic structure. Green-sheet is a composite material that includes inorganic particles of glass, glass-ceramic, ceramic, or mixtures thereof, dispersed in a polymer binder, and may also include additives such as plasticizers and dispersants. The green-sheet is preferably in the form of sheets that are 50 to 250 microns thick. The ceramic

particles are typically metal oxides, such as aluminum oxide or zirconium oxide. An example of such a green-sheet that includes glass-ceramic particles is "AX951" that is sold by E.I. Du Pont de Nemours and Company. An example of a green-sheet that includes aluminum oxide particles is "Ferro Alumina" that is sold by Ferro Corp. The composition of the green-sheet may also be custom formulated to meet particular applications. The green-sheet layers are laminated together and then fired to form a substantially monolithic multilayered structure. The manufacturing, processing, and applications of ceramic green-sheets are described generally in Richard E. Mistler, "Tape Casting: The Basic Process for Meeting the Needs of the Electronics Industry," Ceramic Bulletin, 69(6):1022-1026 (1990), and in U.S. Patent No. 3,991,029, which are incorporated herein by reference.

The method for fabricating devices begins with providing sheets of green-sheet that are preferably 50 to 250 microns thick. The sheets of green-sheet are cut to the desired size, typically 6 inches by 6 inches for conventional processing, although smaller or larger devices may be used as needed. Each green-sheet layer may then be textured using various techniques to form desired structures, such as vias, channels, or cavities, in the finished multilayered structure. In preferred embodiments an array of cavities or wells are constructed. In this preferred embodiment, each well has a micro-electrode in electrical contact with a linking moiety (preferably polyacrylamide gel), wherein the binding molecule is bound to the linking moiety.

Various techniques may be used to texture a green-sheet layer. For example, portions of a green-sheet layer may be punched out to form vias or channels. This operation may be accomplished using conventional multilayer ceramic punches, such as the Pacific Trinetics Corp. Model APS-8718 Automated Punch System. Instead of punching out part of the material, features, such as channels and wells may be embossed into the surface of the green-sheet by pressing the green-sheet against an embossing plate that has a negative image of the desired structure. Texturing may also be accomplished by laser tooling with a laser via system, such as the Pacific Trinetics LVS-3012.

Next, a wide variety of materials may be applied, preferably in the form of thick-film pastes, to each textured green-sheet layer. For example, electrically conductive pathways may be provided by depositing metal-containing thick-film pastes onto the green-sheet layers. Thick-film pastes typically include the desired material, which may be either a metal or a

dielectric, in the form of a powder dispersed in an organic vehicle, and the pastes are designed to have the viscosity appropriate for the desired deposition technique, such as screen-printing. The organic vehicle may include resins, solvents, surfactants, and flow-control agents. The thick-film paste may also include a small amount of a flux, such as a glass frit, to facilitate sintering. Thick-film technology is further described in J.D. Provance, "Performance Review of Thick Film Materials," *Insulation/Circuits* (April, 1977) and in Morton L. Topfer, *Thick Film Microelectronics, Fabrication, Design, and Applications* (1977), pp. 41-59, which are incorporated herein by reference.

The porosity of the resulting thick-film can be adjusted by adjusting the amount of organic vehicle present in the thick-film paste. Specifically, the porosity of the thick-film can be increased by increasing the percentage of organic vehicle in the thick-film paste. Similarly, the porosity of a green-sheet layer can be increased by increasing the proportion of organic binder. Another way of increasing porosity in thick-films and green-sheet layers is to disperse within the organic vehicle, or the organic binder, another organic phase that is not soluble in the organic vehicle. Polymer microspheres can be used advantageously for this purpose.

To add electrically conductive pathways, the thick film pastes typically include metal particles, such as silver, platinum, palladium, gold, copper, tungsten, nickel, tin, or alloys thereof. Silver pastes are preferred. Examples of suitable silver pastes are silver conductor composition numbers 7025 and 7713 sold by E.I. Du Pont de Nemours and Company.

The thick-film pastes are preferably applied to a green-sheet layer by screen-printing. In the screen-printing process, the thick-film paste is forced through a patterned silk screen so as to be deposited onto the green-sheet layer in a corresponding pattern. Typically, the silk screen pattern is created photographically by exposure to a mask. In this way, conductive traces may be applied to a surface of a green-sheet layer. Vias present in the green-sheet layer may also be filled with thick-film pastes. If filled with thick-filled pastes containing electrically conductive materials, the vias can serve to provide electrical connections between layers and/or into wells within an array of wells.

After the desired structures are formed in each layer of green-sheet, preferably a layer of adhesive is applied to either surface of the green-sheet. Preferably, the adhesive is a room-temperature adhesive. Such room-temperature adhesives have glass transition temperatures

below room temperature, *i.e.*, below about 20° C, so that they can bind substrates together at room temperature. Moreover, rather than undergoing a chemical change or chemically reacting with or dissolving components of the substrates, such room-temperature adhesives bind substrates together by penetrating into the surfaces of the substrates. Sometimes such room-temperature adhesives are referred to as "pressure-sensitive adhesives." Suitable room-temperature adhesives are typically supplied as water-based emulsions and are available from Rohm and Haas, Inc. and from Air Products, Inc. For example, a material sold by Air Products, Inc. as "Flexcryl 1653" has been found to work well.

The room-temperature adhesive may be applied to the green-sheet by conventional coating techniques. To facilitate coating, it is often desirable to dilute the supplied pressure-sensitive adhesive in water, depending on the coating technique used, and on the viscosity and solids loading of the starting material. After coating, the room-temperature adhesive is allowed to dry. The dried thickness of the film of room-temperature adhesive is preferably in the range of 1 to 10 microns, and the thickness should be uniform over the entire surface of the green-sheet. Film thicknesses that exceed 15 microns are undesirable. With such thick films of adhesive voiding or delamination can occur during firing due to the large quantity of organic material that must be removed. Films that are less than about 0.5 microns thick when dried are too thin, because they provide insufficient adhesion between the layers of green-sheet.

From among conventional coating techniques, spin-coating and spraying are the preferred methods. If spin-coating is used, it is preferable to add 1 gram of deionized water for every 10 grams of "Flexcryl 1653." If spraying is used, a higher dilution level is preferred to facilitate ease of spraying. Additionally, when room-temperature adhesive is sprayed on, it is preferable to hold the green-sheet at an elevated temperature, e.g., about 60 to 70° C, so that the material dries nearly instantaneously as it is deposited onto the green-sheet. The instantaneous drying results in a more uniform and homogeneous film of adhesive.

After the room-temperature adhesive has been applied to the green-sheet layers, the layers are stacked together to form a multilayered green-sheet structure. Preferably, the layers are stacked in an alignment die, so as to maintain the desired registration between the structures of each layer. When an alignment die is used, alignment holes must be added to each green-sheet layer.

Typically, the stacking process alone is sufficient to bind the green-sheet layers together when a room-temperature adhesive is used. In other words, little or no pressure is required to bind the layers together. However, in order to effect a more secure binding of the layers, the layers are preferably laminated together after they are stacked.

The lamination process involves the application of pressure to the stacked layers. For example, in the conventional lamination process, a uniaxial pressure of about 1000 to 1500 psi is applied to the stacked green-sheet layers that is then followed by an application of an isostatic pressure of about 3000 to 5000 psi for about 10 to 15 minutes at an elevated temperature, such as 70° C. Adhesives do not need to be applied to bind the green-sheet layers together when the conventional lamination process is used.

However, pressures less than 2500 psi are preferable in order to achieve good control over the dimensions of such structures as internal or external cavities and channels. Even lower pressures are more desirable to allow the formation of larger structures, such as cavities and channels. For example, if a lamination pressure of 2500 psi is used, the size of well-formed internal cavities and channels is typically limited to no larger than roughly 20 microns. Accordingly, pressures less than 1000 psi are more preferred, as such pressures generally enable structures having sizes greater than about 100 microns to be formed with some measure of dimensional control. Pressures of less than 300 psi are even more preferred, as such pressures typically allow structures with sizes greater than 250 microns to be formed with some degree of dimensional control. Pressures less than 100 psi, which are referred to herein as "near-zero pressures," are most preferred, because at such pressures, few limits exist on the size of internal and external cavities and channels that can be formed in the multilayered structure.

The pressure is preferably applied in the lamination process by means of a uniaxial press. Alternatively, pressures less than about 100 psi may be applied by hand.

As with semiconductor device fabrication, many devices may be present on each sheet.

Accordingly, after lamination the multilayered structure may be diced using conventional green-sheet dicing or sawing apparatus to separate the individual devices. The high level of peel and shear resistance provided by the room-temperature adhesive results in the occurrence of very little edge delamination during the dicing process. If some layers

become separated around the edges after dicing, the layers may be easily re-laminated by applying pressure to the affected edges, for example by hand, without adversely affecting the rest of the device.

The final processing step is firing to convert the laminated multilayered green-sheet structure from its "green" state to form the finished, substantially monolithic, multilayered structure. The firing process occurs in two important stages as the temperature is raised. The first important stage is the binder burnout stage that occurs in the temperature range of about 250 to 500° C, during which the other organic materials, such as the binder in the green-sheet layers and the organic components in any applied thick-film pastes, are removed from the structure.

The sintering stage, the next important stage of firing, occurs at a higher temperature, in which the inorganic particles sinter together. Sintering results in the multilayered becoming densified and substantially monolithic. The sintering temperature used depends on the nature of the inorganic particles present in the green-sheet. For many types of ceramics, appropriate sintering temperatures range from about 950 to about 1600° C, depending on the material. For example, for green-sheet containing aluminum oxide, sintering temperatures between 1400 and 1600° C are typical. Other ceramic materials, such as silicon nitride, aluminum nitride, and silicon carbide require higher sintering temperatures, namely 1700 to 2200° C. For green-sheet with glass-ceramic particles, a sintering temperature in the range of 750 to 950° C is typical. Glass particles generally require sintering temperatures in the range of only about 350 to 700° C. Finally, metal particles may require sintering temperatures anywhere from 550 to 1700° C, depending on the metal.

Typically, the devices are fired for a period of about 4 hours to about 12 hours or more, depending on the material used. Generally, the firing should be of a sufficient duration so as to remove the organic materials from the structure, and to completely sinter the inorganic particles. In particular, polymers are present as a binder in the green-sheet and in the room-temperature adhesive, and the firing should be of sufficient temperature and duration to decompose these polymers, and to allow for their removal from the multilayered structure.

Typically, the multilayered structure undergoes a reduction in volume during the firing process. During the binder burnout phase, a small volume reduction of about 0.5 to

1.5% is normally observed. At higher temperatures, during the sintering stage, a further volume reduction of about 14 to 17% is typically observed.

The volume change due to firing, on the other hand, can be controlled. In particular, to match volume changes in two materials, such as green-sheet and thick-film paste, one should match: (1) the particle sizes; and (2) the percentage of organic components, such as binders, which are removed during the firing process. Volume changes need not be matched exactly, but any mismatch will typically result in internal stresses in the device. Additionally, symmetrical processing, placing the identical material or structure on opposite sides of the device can, to some extent, compensate for shrinkage stresses caused by mismatched materials. Too great a mismatch in either sintering temperatures or volume changes may result in defects in or failure of some or all of the device. For example, the device may delaminate into its individual layers, or it may become warped or distorted.

Dissimilar materials, such as thick-film pastes or other green-sheet layers, may be added prior to or after the firing process. As noted above, preferably any dissimilar materials added to the green-sheet layers prior to firing, and the dissimilar materials and the green-sheet layers are co-fired. The benefit of co-firing is that the added materials are sintered to the green-sheet layers and become integral to the substantially monolithic microfluidic device. However, to be co-fireable, the added materials should have sintering temperatures and volume changes due to firing that are matched with those of the green-sheet layers. Sintering temperatures are largely material-dependent, so that matching sintering temperatures simply requires proper selection of materials. For example, although silver is the preferred metal for providing electrically conductive pathways, if the green-sheet layers contain alumina particles, which require a sintering temperature in the range of 1400 to 1600° C, some other metal, such as platinum, must be used due to the relatively low melting point of silver (961° C).

Alternatively, the addition of other substrates or joining of two post-sintered (*i.e.* post fired) pieces can be done using any variety of adhesive techniques, including those outlined herein. For example, two "halves" of a device can be glued or fused together. Thus, in a preferred embodiment polyacrylamide gel and biological components, which are not stable at high temperature, can be sandwiched in between the two halves. Alternatively, ceramic

devices comprising open channels or wells can be made, additional substrates or materials placed into the devices, and then they may be sealed with other materials.

The substrates of the invention can form microfluidic cassettes or devices that can be used to effect a number of manipulations on a sample to ultimately result in cell detection or quantification. These manipulations can include cell handling (cell concentration, cell lysis, cell removal, cell separation, etc.), separation of the desired cell from other sample components, chemical or enzymatic reactions on the cell, detection of the cells or other components, etc. The devices of the invention can include one or more wells for sample manipulation, waste or reagents; microchannels to and between these wells, including microchannels containing electrophoretic separation matrices; valves to control fluid movement; on-chip pumps such as electroosmotic, electrohydrodynamic, or electrokinetic pumps; and detection systems. The devices of the invention can be configured to manipulate one or multiple samples or analytes.

The devices of the invention can be made in a variety of ways, as will be appreciated by those in the art. See for example WO96/39260, directed to the formation of fluid-tight electrical conduits; U.S. Patent No. 5,747,169, directed to sealing; EP 0637996 B1; EP 0637998 B1; WO96/39260; WO97/16835; WO98/13683; WO97/16561; WO97/43629; WO96/39252; WO96/15576; WO96/15450; WO97/37755; and WO97/27324; and U.S. Patent Nos. 5,304,487; 5,071531; 5,061,336; 5,747,169; 5,296,375; 5,110,745; 5,587,128; 5,498,392; 5,643,738; 5,750,015; 5,726,026; 5,35,358; 5,126,022; 5,770,029; 5,631,337; 5,569,364; 5,135,627; 5,632,876; 5,593,838; 5,585,069; 5,637,469; 5,486,335; 5,755,942; 5,681,484; and 5,603,351, all of which are hereby incorporated by reference. Suitable fabrication techniques again will depend on the choice of substrate, but preferred methods include, but are not limited to, a variety of micromachining and microfabrication techniques, including film deposition processes such as spin coating, chemical vapor deposition, laser fabrication, photolithographic and other etching techniques using either wet chemical processes or plasma processes, embossing, injection molding and bonding techniques (see U.S. Patent No. 5,747,169, hereby incorporated by reference). In addition, there are printing techniques for the creation of desired fluid guiding pathways; that is, patterns of printed material can permit directional fluid transport. Thus, the build-up of "ink" can serve to define a flow channel. In addition, the use of different "inks" or "pastes" can allow different

portions of the pathways having different flow properties. For example, materials can be used to change solute/solvent RF values (the ratio of the distance moved by a particular solute to that moved by a solvent front). For example, printed fluid guiding pathways can be manufactured with a printed layer or layers comprised of two different materials, providing different rates of fluid transport. Multi-material fluid guiding pathways can be used when it is desirable to modify retention times of reagents in fluid guiding pathways. Furthermore, printed fluid guiding pathways can also provide regions containing reagent substances, by including the reagents in the "inks" or by a subsequent printing step. See for example U.S. Patent No. 5,795,453, herein incorporated by reference in its entirety.

In addition, it should be understood that while most of the discussion herein is directed to the use of planar substrates with microchannels and wells, other geometries can be used as well. For example, two or more planar substrates can be stacked to produce a three dimensional device, that can contain microchannels flowing within one plane or between planes; similarly, wells may span two or more substrates to allow for larger sample volumes. Thus for example, both sides of a substrate can be etched to contain microchannels; see for example U.S. Patent Nos. 5,603,351 and 5,681,484, both of which are hereby incorporated by reference.

Thus, the devices of the invention may include at least one microchannel or flow channel that allows the flow of sample from the sample inlet port to the other components or modules of the system. The collection of microchannels and wells is sometimes referred to in the art as a "mesoscale flow system". As will be appreciated by those in the art, the flow channels may be configured in a wide variety of ways, depending on the use of the channel. For example, a single flow channel starting at the sample inlet port may be separated into a variety of smaller channels, such that the original sample is divided into discrete subsamples for parallel processing or analysis. Alternatively, several flow channels from different modules, for example the sample inlet port and a reagent storage module may feed together into a mixing chamber or a reaction chamber. As will be appreciated by those in the art, there are a large number of possible configurations; what is important is that the flow channels allow the movement of sample and reagents from one part of the device to another. For example, the path lengths of the flow channels may be altered as needed; for example, when

mixing and timed reactions are required, longer and sometimes tortuous flow channels can be used.

In some embodiments, the devices herein are designed on a scale suitable to analyze microvolumes, although in some embodiments large samples (e.g. cc's of sample) may be reduced in the device to a small volume for subsequent analysis. That is, "mesoscale" as used herein refers to chambers and microchannels that have cross-sectional dimensions on the order of 0.1 μm to 500 μm. The mesoscale flow channels and wells have preferred depths on the order of 0.1 μm to 100 μm, typically 2-50 μm. The channels have preferred widths on the order of 2.0 to 500 μm, more preferably 3-100 μm. For many applications, channels of 5-50μm are useful. However, for many applications, larger dimensions on the scale of millimeters may be used. Similarly, chambers (sometimes also referred to herein as "wells") in the substrates often will have larger dimensions, on the scale of a few millimeters. In addition to the flow channel system, the devices of the invention are configured to include one or more of a variety of components, herein referred to as "modules", that will be present on any given device depending on its use. These modules include, but are not limited to: sample inlet ports; sample introduction or collection modules; cell handling modules (for example, for cell lysis, cell removal, cell concentration, cell separation or capture, cell growth, etc.); separation modules, for example, for electrophoresis, dielectrophoresis, gel filtration, ion exchange/affinity chromatography (capture and release) etc.; reaction modules for chemical or biological alteration of the sample, including amplification of the nucleic acids, chemical, physical or enzymatic cleavage or alteration of the sample components, or chemical modification of the target; fluid pumps; fluid valves; thermal modules for heating and cooling; storage modules for assay reagents; mixing chambers; and detection modules.

In a preferred embodiment, the devices of the invention include at least one sample inlet port for the introduction of the sample to the device. This may be part of or separate from a sample introduction or collection module; that is, the sample may be directly fed in from the sample inlet port to a separation chamber, or it may be pretreated in a sample collection well or chamber.

In a preferred embodiment, the devices of the invention include a sample collection module, which can be used to concentrate or enrich the sample if required; for example, see

U.S. Patent No. 5,770,029, including the discussion of enrichment channels and enrichment means.

In a preferred embodiment, the devices of the invention include a cell handling module. Thus, for example, the detection of particular antibodies in blood can require the removal of the blood cells for efficient analysis, or the cells (and/or nucleus) may be lysed. In this context, "cells" include eukaryotic and prokaryotic cells, and viral particles that may require treatment prior to analysis, such as the release of nucleic acid from a viral particle prior to detection of target sequences. In addition, cell handling modules may also utilize a downstream means for determining the presence or absence of cells. Suitable cell handling modules include, but are not limited to, cell lysis modules, cell removal modules, cell concentration modules, and cell separation or capture modules. In addition, as for all the modules of the invention, the cell handling module is in fluid communication via a flow channel with at least one other module of the invention.

In a preferred embodiment, the cell handling module includes a cell lysis module. As is known in the art, cells may be lysed in a variety of ways, depending on the cell type. In one embodiment, as described in EP 0 637 998 B1 and U.S. Patent No. 5,635,358, hereby incorporated by reference, the cell lysis module may comprise cell membrane piercing protrusions that extend from a surface of the cell handling module. As fluid is forced through the device, the cells are ruptured. Similarly, this may be accomplished using sharp edged particles trapped within the cell handling region. Alternatively, the cell lysis module can comprise a region of restricted cross-sectional dimension, which results in cell lysis upon pressure.

In a preferred embodiment, the cell lysis module comprises a cell lysing agent, such as guanidium chloride, chaotropic salts, enzymes such as lysozymes, etc. In some embodiments, for example for blood cells, a simple dilution with water or buffer can result in hypotonic lysis. The lysis agent may be solution form, stored within the cell lysis module or in a storage module and pumped into the lysis module. Alternatively, the lysis agent may be in solid form, that is taken up in solution upon introduction of the sample.

The cell lysis module may also include, either internally or externally, a filtering module for the removal of cellular debris as needed. This filter may be microfabricated between the cell lysis module and the subsequent module to enable the removal of the lysed

cell membrane and other cellular debris components; examples of suitable filters are shown in EP 0 637 998 B1, incorporated by reference.

In a preferred embodiment, the cell handling module includes a cell separation or capture module. This embodiment utilizes a cell capture region comprising binding sites capable of reversibly binding a cell surface molecule to enable the selective isolation (or removal) of a particular type of cell from the sample. These binding moieties may be immobilized either on the surface of the module or on a particle trapped within the module (i.e. a bead) by physical absorption or by covalent attachment. Suitable binding moieties will depend on the cell type to be isolated or removed, and generally includes antibodies and other binding ligands, such as ligands for cell surface receptors, etc. as outlined herein Thus, a particular cell type may be removed from a sample prior to further handling, or the assay is designed to specifically bind the desired cell type, wash away the non-desirable cell types, followed by either release of the bound cells by the addition of reagents or solvents, physical removal (i.e. higher flow rates or pressures), or even in situ lysis.

Alternatively, a cellular "sieve" can be used to separate cells on the basis of size. This can be done in a variety of ways, including protrusions from the surface that allow size exclusion, a series of narrowing channels, a weir, or a diafiltration type setup.

In a preferred embodiment, the cell handling module includes a cell removal module. This may be used when the sample contains cells that are not required in the assay or are undesirable. Generally, cell removal will be done on the basis of size exclusion as for "sieving", above, with channels exiting the cell handling module that are too small for the cells.

In a preferred embodiment, the cell handling module includes a cell concentration module. As will be appreciated by those in the art, this is done using "sieving" methods, for example to concentrate the cells from a large volume of sample fluid prior to lysis.

In some embodiments of the present invention, the microelectrodes project from the surface of the substrate, with such projections extending between 5×10^{-8} and 1×10^{-5} cm from the surface of the supporting substrate. In other embodiments, the microelectrodes comprise a flat disk of conductive material that is embedded in the supporting substrate and is exposed at the substrate surface, with the supporting substrate acting as an insulator in the spaces between the microelectrodes.

In a preferred embodiment of the present invention the microelectrodes comprise a gold or platinum conductor and a glass or silicon insulator. In alternative embodiments, the microelectrodes comprise conductor substances such as solid or porous foils or films of silver, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof. In additional embodiments, the microelectrodes comprise substrate and/or insulator substances such as plastic, rubber, fabric, ceramics, or combinations thereof. The microelectrodes of the present invention preferably have an exposed conductive surface of from about 0.01μm^2 to 0.5 cm^2. In a preferred embodiment, the exposed conductive material has an area of from about 100 to 160,000μm^2.

One embodiment of the present invention is shown in Figure 1A, wherein the microelectrode comprises a glass capillary tube 1, containing an ultra fine platinum wire 2, to which a transition wire 3 has been soldered 6. The transition wire 3 is soldered 6 in turn to a hookup wire 4, which protrudes from an epoxy plug 5 that seals the capillary tube. Polyacrylamide gel material 7 is packed into a recess etched into the exposed surface of the platinum wire 2. The polymeric hydrogel pad is preferably at least about 0.1 to 30 μm thick, more preferably at least about 0.5 to 10μm thick, and most preferably about 0.5μm thick.

The apparatus of the present invention comprises at least one counter-electrode. In a preferred embodiment of the present invention, the counter-electrode comprises a conductive material, with an exposed surface that is significantly larger than that of the individual microelectrodes. In a preferred embodiment, the counter electrode comprises platinum wire. In altemative embodiments, the counter electrode comprises solid or porous films of gold, silver, platinum, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof.

In other embodiments of the present invention, the apparatus comprises at least one reference electrode. The reference electrode is particularly useful in assays for detecting the change in electrical potential after probe and target molecules are allowed to interact and then determining the number or concentration of bacterial cells in a sample using quantitative methods (e.g., voltammetry). In preferred embodiments, the reference electrode comprises a silver/silver chloride electrode. In altemative embodiments, the reference electrode comprises solid or porous films of gold, platinum, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof.

In still further embodiments of the present invention, the apparatus further comprises a plurality of wells each of which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode. The term "wells" is used herein in its conventional sense, to describe a portion of the supporting substrate in which the microelectrode and at least one counter-electrode are contained in a defined volume; said wells can protrude from the surface of the supporting substrate, or be embedded therein.

Electrochemical contact between each of the microelectrodes and the counter electrode and/or the reference electrode is advantageously provided using an electrolyte solution in contact with each of the microelectrodes comprising the apparatus of the invention. Electrolyte solutions useful in the apparatus and methods of the invention include any electrolyte solution at physiologically-relevant ionic strength (equivalent to about 0.15 M NaCl) and neutral pH. Examples of electrolyte solutions useful with the apparatus and methods of the invention include, but are not limited to, phosphate buffered saline, HEPES buffered solutions, and sodium bicarbonate buffered solutions. Said electrolyte solutions are in contact with each of the microelectrodes of the apparatus of the invention, the counter-electrode and the reference electrode if provided, thereby providing electrochemical contact between the electrodes.

In preferred embodiments of the present invention, molecular interactions between probe molecules immobilized on linker moieities in contact with microelectrodes and proetin or peptide target molecules in a sample mixture are detected by detecting an electrical signal using AC impedance. In other embodiments, such molecular interactions are detected by detecting an electrical signal using an lelectrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods, and combinations thereof.

In one embodiment of the apparatus of the present invention, the means for producing electrical impedance at each microelectrode is accomplished using a Model 1260 Impedance/Gain-Phase Analyser with Model 1287 Electrochemical Interface (Solartron Inc., Houston, TX). Other electrical impedance measurement means include, but are not limited

to, transient methods using AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell such as AC bridge and AC voltammetry. The measurements can be conducted at a certain particular frequency that specifically produces electrical signal changes that are readily detected or otherwise determined to be advantageous. Such particular frequencies are advantageously determined by scanning frequencies to ascertain the frequency producing, for example, the largest difference in electrical signal. The means for detecting changes in impedance at each microelectrode as a result of antibody-bacterial cell binding can be accomplished by using any of the above-described instruments and analytical methods.

In a preferred embodiment of the present invention the target molecule is a protein (for example and without limitation an antigen, receptor, or ligand), which is detected in a sample using microelectrodes in contact with linker moieties to which binding ligands capable of specifically binding to the target protein (antibodies, small molecules, proteins, peptides, polypeptides, or aptamers) have been immobilized. In one method of the invention, AC impedance is measured at a plurality of microelectrodes in contact with linker moieties to which a probe having specificity to the target molecule has been immobilized. The microelectrodes are then exposed to a sample mixture containing a sample mixture containing the target molecule, and changes in AC impedance resulting from molecular interactions between the target molecules and binding ligands are then detected at each of the microelectrodes.

In some embodiments electrochemical reporters are used to enhance the detection of target molecules. For example and without limitation, electrochemical reporters ("reporter group") is attached to the target molecule. Reporters are electrochemically-distinctive redox moieties, or an electron transfer moiety that do not interfere with the molecular interaction to be detected. Electrochemically-labeled target molecules are electrochemically active, *i.e.*., they are capable of participating in oxidation/reduction reaction under conditions of applied voltage potential, and are compatible with the probes, target, and solution carrying the target. Electrochemically-labeled target molecules useful in the methods of the present invention may be prepared by labeling suitable target molecules with any reporter group having an electrochemically-distinctive property, most preferably a redox (oxidation/reduction)

potential that can be distinguished from other components of the binding reaction, and that does not interfere with the molecular interaction to be detected.

As used herein "electron transfer moiety", "ETM", or grammatical equivalents thereof means a compound or group that is capable of reversibly, semi-reversibly, or irreversibly transferring one or more electrons. As used herein "electron donor moiety", "electron acceptor moiety", "electron transfer moieties" or grammatical equivalents thereof refers to molecules capable of electron transfer under certain conditions, for example oxidation-reduction reactions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred electron transfer moieties include, but are not limited to, transition metal complexes, organic electron transfer moieties, and electrodes.

In preferred embodiments target molecules are labeled with reporters comprising a transition metal complex or an organic redox couple. Transition metals include those whose atoms have a partial or complete d shell of electrons; elements having the atomic numbers 21-30, 39-48, 57-80 and the lanthanide series. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

The transition metals are complexed with a variety of ligands, generally depicted herein as "L", to form suitable transition metal complexes, as is well known in the art. Suitable ligands fall into two categories: ligands which use nitrogen, oxygen, sulfur, carbon or phosphorus atoms (depending on the metal ion) as the coordination atoms (generally referred to in the literature as sigma (σ) donors) and organometallic ligands such as

metallocene ligands (generally referred to in the literature as pi (π) donors, and depicted herein as L_m). Suitable nitrogen donating ligands are well known in the art and include, but are not limited to, NH_2; NHR; NRR'; pyridine; pyrazine; isonicotinamide; imidazole; bipyridine and substituted derivatives of bipyridine; terpyridine and substituted derivatives; phenanthrolines, particularly 1,10-phenanthroline (abbreviated phen) and substituted derivatives of phenanthrolines such as 4,7-dimethylphenanthroline and dipyridol[3,2-a:2',3'-c]phenazine (abbreviated dppz); dipyridophenazine; 1,4,5,8,9,12-hexaazatriphenylene (abbreviated hat); 9,10-phenanthrenequinone diimine (abbreviated phi); 1,4,5,8-tetraazaphenanthrene (abbreviated tap); 1,4,8,11-tetra-azacyclotetradecane (abbreviated cyclam) and isocyanide. Substituted derivatives, including fused derivatives, may also be used. In some embodiments, porphyrins and substituted derivatives of the porphyrin family may be used. *See, e.g.*, Comprehensive Coordination Chemistry, Ed. Wilkinson *et al.*, Pergammon Press, 1987, Chapters 13.2 (pp73-98), 21.1 (pp. 813-898) and 21.3 (pp 915-957), all of which are hereby expressly incorporated by reference.

Suitable sigma donating ligands using carbon, oxygen, sulfur and phosphorus are known in the art. For example, suitable sigma carbon donors are found in Cotton and Wilkenson, Advanced Organic Chemistry, 5th Edition, John Wiley & Sons, 1988, hereby incorporated by reference; see page 38, for example. Similarly, suitable oxygen ligands include crown ethers, water and others known in the art. Phosphines and substituted phosphines are also suitable; see page 38 of Cotton and Wilkenson. The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used as reporters. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ-bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (*see, e.g.*, Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich *et al.*, 2nd Ed., 1992; VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel *et al.* Ed., Vol. 7, chapters 7, 8, 10 & 11, Pergamon Press, all hereby expressly incorporated by reference). Such organometallic

ligands include cyclic aromatic compounds such as the cyclopentadienide ion [$C_5H_5(-1)$] and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl)metal compounds, (i.e. the metallocenes). *See, e.g.,* Robins *et al.*, J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman *et al.*, J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene [$(C_5H_5)_2Fe$] and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly *et al.*, Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger *et al.*, Advances in Organometallic Chemistry 23:1-93; and Geiger *et al.*, Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example, Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjuction with other π-bonded and δ-bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties.

In addition to transition metal complexes, other organic electron donors and acceptors may be used in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, *N,N'*-dimethyl-2,7-diazapyrenium dichloride (DAP^{2+}), methylviologen, ethidium bromide, quinones such as N,N'-dimethylanthra(2,1,9-*def*:6,5,10-*d'e'f'*)diisoquinoline dichloride ($ADIQ^{2+}$); porphyrins ([meso-tetrakis(N-methyl-x-pyridinium)porphyrin tetrachloride], varlamine blue B hydrochloride, Bindschedler's green; 2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue; Nile blue A (aminoaphthodiethylaminophenoxazine sulfate), indigo-5,5',7,7'-tetrasulfonic acid, indigo-5,5',7-trisulfonic acid; phenosafranine, indigo-5-monosulfonic acid; safranine T; bis(dimethylglyoximato)-iron(II) chloride; induline scarlet, neutral red,

anthracene, coronene, pyrene, 9-phenylanthracene, rubrene, binaphthyl, DPA, phenothiazene, fluoranthene, phenanthrene, chrysene, 1,8-diphenyl-1,3,5,7-octatetracene, naphthalene, acenaphthalene, perylene, TMPD and analogs and subsitituted derivatives of these compounds.

In other embodiments of the present invention, molecules are labeled with the following non-limiting examples of electrochemically-active moieties: 1,4-benzoquinone, ferrocene, tetracyanoquinodimethane, N,N,N',N'-tetramethyl p-phenylenediamine, tetrathiafulvalene, 9-aminoacridine, acridine orange, aclarubicin, daunomycin, doxorubicin, pirarubicin, ethidium bromide, ethidium monoazide, chlortetracycline, tetracycline, minocycline, Hoechst 33258, Hoechst 33342, 7-aminoactinomycin D, Chromomycin A_3, mithramycin A, Vinblastine, Rifampicin, $Os(bipyridine)_2(dipyridophenazine)_2^+$, $Co(bipyridine)_3^{3+}$, or Fe-bleomycin.

The electrochemically-active moiety comprising the electrochemically active reporter-labeled molecule used in certain embodiments of the methods of the present invention is optionally linked to the antibody molecule through a linker, preferably having a length of from about 10 to-about 20 Angstroms. The linker can be an organic moiety such as a hydrocarbon chain $(CH_2)_n$, wherein n is an integer from 1 to about 20, or can comprise an ether; ester, carboxyamide, or thioether moiety, or a combination thereof. The linker can also be an inorganic moiety such as siloxane (O-Si-O). The length of the linker is selected so that the electrochemically-active moiety does not interfere with the molecular interaction to be detected.

"Competition assays" may be performed in accordance with an alternative embodiment of the present invention. In this embodiment a known amount of a competitor molecule labeled with an electrochemical reporter is exposed to an array of immobilized probe molecules, most preferably before a reaction mixture containing an unlabeled target molecule is exposed to the array. The amount of target molecule present in the reaction mixture is then determined indirectly by measuring the amount of labeled competitor molecule that is displaced from the array following the addition of the reaction mixture. "Sandwich assays", in which a target binding molecule labeled with an electrochemical reporter is exposed to an array of immobilized probe molecules following exposure of the array to a reaction mixture, may be performed in accordance with another alternative

embodiment of the present invention. The target binding molecule is selected for its ability to interact with the target molecule without interfering with the interaction between the target molecules and the immobilized probe molecules. The "sandwich" assay is particularly useful for detecting target molecules having higher molecular weights and results in an increase in experimental specificity and sensitivity.

The preferred embodiments of the present invention are best understood by referring to Figures 1-6 and Examples 1-3. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLE 1

Preparation of Streptavidin-Modified Porous Hydrogel Microelectrodes

Streptavidin-modified porous hydrogel microelectrodes used for detecting viable bacteria were prepared as follows. Ultra-fine platinum wire having a diameter of 50μm was inserted into glass capillary tubing having a diameter of 2 mm and sealed by heating to form a solidmicroelectrode structure. The tip of the structure was then polished with gamma alumina powder (CH Instruments, Inc., Austin, TX) to expose a flat disk of the platinum wire. Microelectrodes were initially polished with 0.3 μm gamma alumina powder, rinsed with deionized water, and then polished with 0.005μm powder. Following polishing, the microelectrodes were ultrasonically cleaned for 2 min. in deionized water, soaked in 1N HNO_3 for 20 min., vigorously washed in deionized water, immersed in acetone for 10 min., and again washed vigorously in deionized water. Through the use of micromanufacturing techniques employed in the fabrication of semiconductors, modifications of this procedure can be applied to the preparation of microelectrodes of a size required for the construction of alternative embodiments of the microelectrodes, such as bioarray chips (see co-owned and co-pending U.S. Patent App. Serial Nos. 09/458,501 and 09/458,533, incorporated by reference).

Porous hydrogel microelectrodes were prepared from the above-described microelectrodes as follows. The exposed flat disk of platinum of each microelectrode was etched in hot aqua regia to form a recess (*i.e.*, micropore dent) of a specified depth. The depth of the recess was controlled by the length of time that the platinum disk was exposed to

the etching material, with the depth of the micropore dent ranging from several microns to more than 1mm. The recess thus formed was then packed with 1μL of streptavidin-modified polyacrylamide gel material (Figure 1B) and polymerized under UV irradiation in a Stratalinker (Stratagene, La Jolla, CA) for 20 min. to form a porous hydrogel microelectrode (Figure 2). A porous hydrogel microelectrode having a diameter of about 260μm was used as described below in Example 2. Prior to attachment of antibodies, porous hydrogel microelectrodes were ultrasonically treated in acetone and then in 1M HNO_3 for 10 min.

Streptavidin-modified polyacrylamide gel material was prepared as follows. A streptavidin solution was prepared by mixing 10 μ L of an N-acyloxysuccinimide solution (prepared by dissolving 10 mg of N-acyloxysuccinimide in 72μL of DMSO) with 200μL of streptavidin stock solution (prepared by dissolving 10 mg streptavidin in 2.5 mL phosphate buffered saline (PBS) at pH 7.6). This mixture was incubated at room temperature for 2-3 hours and then centrifuged for 2 minutes at 13,000 rpm in a conventional desktop eppendorf microcentrifuge to remove precipitated material. An acrylamide solution was prepared by first dissolving 25 mg bis-acrylamide in 6 mL PBSt pH 7.6, dissolving 475 mg acrylamide monomer in this solution, and then filtering the mixture through a 5 micron filter. To prepare streptavidin-modified polyacrylamide gel material, 290 ~L of the acrylamide solution was mixed with 210μL of the streptavidin solution, and 150μL of this solution was added to 0.6μL of 1mM methylene blue and 1.8μL TEMED, poured into the recesses in the microelectrodes and allowed to polymerize prior to use.

EXAMPLE 2

Immobilization of Antibodies on Streptavidin-Modified Porous Hydrogel Microelectrodes

To attach antibodies to the microelectrodes prepared in Example 1, the microelectrodes were incubated at room temperature or at about 25°C for 1.5 hours in 500μL of a solution consisting of 50μg/mL of biotinylated polyclonal anti *E. coli* antibody (Virostat, Portland, ME) having specificity for a plurality of *E. coli* antigens. Following attachment of the antibodies, the microelectrodes were vigorously washed in PBS prior to use.

EXAMPLE 3

Electrical Detection of Antibody-Antigen Interactions

Molecular interactions between antibodies immobilized on a porous hydrogel microelectrode and antigens in a sample solution were detected using the porous hydrogel microelectrodes described herein to measure changes in AC impedance. AC impedance was measured using a Model 1260 Impedance/Gain-Phase Analyser with Model 1287 Electrochemical Interface (Solartron Inc.). A platinum wire having a surface area larger than the porous hydrogel microelectrode was used as the counter-electrode. Impedance measurements were made under open circuit voltage (OCV) conditions in PBS and samples were excited at an amplitude of 20 mV.

Using streptavidin-modified porous hydrogel microelectrodes prepared as described in Example 1, baseline AC impedance was measured in PBS. Following incubation of microelectrodes in anti-BAP antibody as described in Example 2, AC impedance was measured again. Microelectrodes were then incubated in a 50μg/mL BAP antigen solution (Sigma, St. Louis, MO) at about 4°C for 15 hours. Following vigorous rinsing in PBS, AC impedance was once again measured. Figures 3A and 3B illustrate plots of capacitance versus frequency (Figure 3A) and resistance versus frequency (Figure 3B) for microelectrodes before (curve 1) and after (curve 2) immobilization of rabbit anti-BAP antibody, and follou ing incubation with BAP antigen (curve 3). These plots indicate that, following immobilization of anti-BAP antibody, there was an increase in capacitance and a decrease in resistance, and following interaction with BAP antigen, there was a further increase in capacitance and a further decrease in resistance. These results suggest that molecular interactions between an antibody probe immobilized on porous hydrogel microelectrodes and antigen target molecules in a sample solution can be detected by examining changes in AC impedance.

To examine whether molecular interactions between an immobilized antibody and secondary antibody could be electrically detected, microelectrodes with anti-BAP antibody-BAP antigen complexes were allowed to interact with fluorescein-labeled antirabbit IgG antibody (Boehringer-Mannheim, Indianapolis, IN) at a concentration of 40μg/mL at 4°C for 24 hours. Following this incubation, AC impedance was once again measured. Figures 4A and 4B illustrate plots of capacitance versus frequency (Figure 4A) and resistance v ersus

frequency (Figure 4B) for microelectrodes with immobilized rabbit anti-BAP antibody before (curve 1) and after (curve 2) incubation with BAP antigen, and following incubation with an anti-rabbit IgG secondary antibody (curve 3). These plots indicate that. follouing interaction with the secondary antibody, there was a further increase in capacitance and a further decrease in resistance over that detected for the primary antibody-antigen complex alone. These results suggest that molecular interactions between antibody-antigen complexes immobilized on porous hydrogel microelectrodes and secondary antibody target molecules in a sample solution can be detected by examining changes in AC impedance.

In addition, these results indicate that the present invention can be used to create immunoassay sensors utilizing antibodies capable of recognizing and binding the target molecule to increase the signal and improve specificity. The apparatus and methods of the present invention, for example, would be useful for detecting antibodies to an infectious agent in immunoassays of serum. The specificity and sensitivity of such an assay would be increased by the application of a specific capture antibody (*i.e.*, the immobilized probe) and a second target molecule binding antibody, as described herein.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

What is claimed is:

1. An apparatus for the electrical detection of molecular interactions between an immobilized probe molecule and a protein or peptide target molecule. comprising:

(a) a supporting substrate;

(b) one or a plurality of microelectrodes in contact with the supporting substrate;

(c) one or a plurality of linking moieties in contact with the microelectrodes and to which probe molecules have been immobilized;

(d) at least one counter-electrode in electrochemical contact with the microelectrodes;

(e) a means for producing an electrical signal at each microelectrode;

(f) a means for detecting changes in the electrical signal at each microelectrode; and

(g) an electrolyte solution in contact with the microelectrodes. the linking moieties, and the counter-electrode, wherein molecular interactions between the immobilized probe molecules and protein or peptide target molecule are detected as a difference in the electrical signal at each microelectrode in the presence and absence of the protein or peptide target molecule.

2. The apparatus of Claim 1, w herein the linking moieties comprise polyacrylamide gel, agarose gel, polyethylene glycol, cellulose gel, sol gel, or combinations thereof.

3. The apparatus of Claim 2, wherein the linking moieties comprise polyacrylamide gel.

4. The apparatus of Claim 1. w herein the linking moieties comprise a conjugated polymer or copolymer film.

5. The apparatus of Claim 4, wherein the conjugated polymer or copolymer film is polypyrrole, polythiphene, polyaniline, polyfuran, polypyridine, polycarbazole, polyphenylene, poly(phenylenvinylene), polyfluorene, or polyindole, or their derivatives copolymers, or combinations thereof.

6. The apparatus of Claim 1, wherein the linking moieties comprise a neutral pyrrole matrix.

7. The apparatus of Claim 1, wherein the supporting substrate comprises ceramic, glass, silicon, silicon nitride, fabric, rubber, plastic, printed circuit board, or combinations thereof.

8. The apparatus of Claim 1, wherein the microelectrodes comprise a conductive material and an insulating material.

9. The apparatus of Claim 8, wherein the conductive material is solid or porous gold, silver, platinum, titanium, copper, chromium, or aluminum, or metal oxide, metal nitride, metal carbide, carbon, graphite, conductive plastic, metal impregnated polymers or combinations thereof.

10. The apparatus of Claim 9, wherein the conductive material is platinum.

11. The apparatus of Claim 9, wherein the conductive material is gold.

12. The apparatus of Claim 8, wherein the insulating material is glass, silicon, silicon nitride, plastic, rubber, fabric, ceramic, printed circuit board, or combinations thereof.

13. The apparatus of Claim 12, wherein the insulating material is silicon.

14. The apparatus of Claim 12, wherein the insulating material is glass.

15. The apparatus of Claim 8, wherein the conductive material is embedded the supporting substrate and the supporting substrate comprises the insulating material.

16. The apparatus of Claim 1, further comprising at least one reference electrode.

17. The apparatus of Claim 16. wherein the reference electrode comprises a conductive material and an insulating material.

18. The apparatus of Claim 17, wherein the conductive material is solid or porous gold, silver, platinum, titanium, copper, chromium, or aluminum, or metal oxide, metal nitride, metal carbide, carbon, graphite, conductive plastic, metal impregnated polymers or combinations thereof.

19. The apparatus of Claim 17. wherein the conductive material is silver/silver chloride.

20. The apparatus of Claim 17, wherein the insulating material is glass, silicon, silicon nitride, plastic, rubber, fabric. ceramic. printed circuit board, or combinations thereof.

21. The apparatus of Claim 1, wherein the supporting substrate further comprises a plurality of wells, each of which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode.

22. The apparatus of Claim 1. wherein the probe molecules are oligonucleotides or nucleic acids.

23. The apparatus of Claim 22, wherein the probe molecules are aptamers.

24. The apparatus of Claim 1, wherein the probe molecules are proteins or peptides.

25. The apparatus of Claim 24, wherein the probe molecules are antibodies.

26. The apparatus of Claim 25, wherein the antibodies are polyclonal antisera, polyclonal antibodies, or F(ab), F(ab)', $F(ab)_2$, or F_v fragments thereof.

27. The apparatus of Claim 25, wherein the antibodies are monoclonal antibodies, or F(ab), F(ab)', $F(ab)_2$, or F_v fragments thereof.

28. The apparatus of Claim 25, wherein the antibodies are F(ab) fragments or single-chain F_v fragments produced by in vitro libraries.

29. The apparatus of Claim 1, wherein the probe molecules comprise a natural products library, a peptide library. a phage display library, or a combinatorial library.

30. The apparatus of Claim 1, wherein the linking moieties further comprise streptavidin and the probe molecules are biotinylated.

31. The apparatus of Claim 1, wherein molecular interactions between probe molecules and protein or peptide target molecules are detected by using an electrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements. current step method, other steady-state or transient measurement methods, and combinations thereof.

32. A method for the electrical detection of molecular interactions between an immobilized probe molecule and a protein or peptide target molecule. comprising:

(a) detecting a first electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized;

(b) exposing the one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized to a sample mixture containing protein or peptide target molecules;

(c) detecting a second electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized;

(d) comparing the first electrical signal with the second electrical signal, and

(e) determining whether the first electrical signal is different from the second electrical signal.

33. The method of Claim 32. wherein molecular interactions between probe molecules and protein or peptide target molecules are detected by using an electrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry. square wave voltammetry, AC voltammetry, hydrodynamic

modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods, and combinations thereof.

34. The method of Claim 32, wherein the electrical detection method is AC impedance that is measured over a range of frequencies.

35. The method of Claim 32, wherein the electrical detection method is AC impedance that is measured by transient methods with AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell.

36. The method of Claim 32, wherein the electrical detection method is AC impedance that is measured by impedance analyzer. lock-in amplifier, AC bridge, AC voltammetry, or combinations thereof.

37. The method of Claim 32, wherein the linker moieties comprise polyacrylamide gel, agarose gel. polyethylene glycol. cellulose gel. sol gel, or combinations thereof and the protein or peptide target molecules are labeled with an electrochemically active reporter molecule prior to exposing the one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized to the sample mixture containing protein or peptide target molecules.

38. The method of Claim 37, wherein the electrochemically active reporter molecule comprises a transition metal complex.

39. The method of Claim 37, wherein the transition metal ion is ruthenium, cobalt, iron. zinc, copper, magnesium, nickel, or osmium.

40. The method of Claim 37, wherein the electrochemically active reporter labeled target molecules are labeled with electrochemical reporter groups selected from the group

consisting of 1,4-benzoquinone, ferrocene, tetracyanoqublodimethane, N,N,N',N'-tetramethyl-p-phenylenediamine, and tetrathiafulvalene.

41. The method of Claim 37, wherein the electrochemically active reporter labeled target molecules are labeled with electrochemical reporter groups selected from the group consisting of 9-aminoacridine, acridine orange, aclarubicin, daunomycin, doxorubicin, pirarubicin, ethidium bromide, ethidium monoazide, chlortetracycline, tetracycline, minocycline, Hoechst 33258, Hoechst 33342, 7-aminoactinomycin D, Chromomycin A_3, mithramycin A, Vinblastine, Rifampicin, $Os(bipyridine)_2(dipyridophenazine)_2^+$, $Co(bipyridine)_3^{3+}$, and Fe-bleomycin.

42. The method of Claim 32, further comprising:

(f) exposing the one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized to an electrochemically labeled target binding molecule;

(g) detecting a third electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized.

(h) comparing the second electrical signal with the third electrical signal, and

(i) determining whether the second electrical signal is different from the third electrical signal.

43. The method of Claim 42, wherein the target binding molecules are oligonucleotides or nucleic acids.

44. The method of Claim 43, wherein the target binding molecules are aptamers.

45. The method of Claim 42. wherein the target binding molecules are proteins or peptides.

46. The method of Claim 45. wherein the target binding molecules are antibodies.

47. The method of Claim 46, wherein the antibodies are polyclonal antisera, polyclonal antibodies, or F(ab), F(ab)', $F(ab)_2$, or F_v fragments thereof.

48. The method of Claim 46, wherein the antibodies are monoclonal antibodies, or F(ab), F(ab)', $F(ab)_2$,or F_v fragments thereof.

49. The method of Claim 46, wherein the antibodies are F(ab) fragments or single-chain F_v fragments produced by *in vitro* libraries.

50. The method of Claim 42, wherein the target binding molecules are a natural products library, a peptide library, a phage display library, or a combinatorial library.

51. A method for the electrical detection of molecular interactions between an immobilized probe molecule and a protein or peptide target molecule, comprising:

(a) detecting a first electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized;

(b) exposing the one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized to a sample mixture containing a first protein or peptide target molecule and to a second protein or peptide target molecule, wherein the second protein or peptide target molecule is labeled with an electrochemical reporter;

(c) detecting a second electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which probe molecules have been immobilized;

(d) comparing the first electrical signal with the second electrical signal; and

(e) determining whether the first electrical signal is different from the second electrical signal.

FIG. 1A

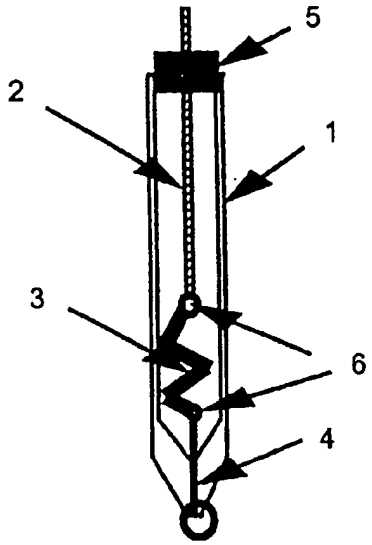


FIG. 1B

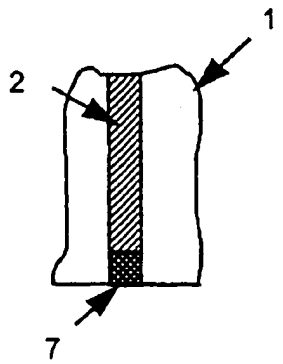


FIG. 2

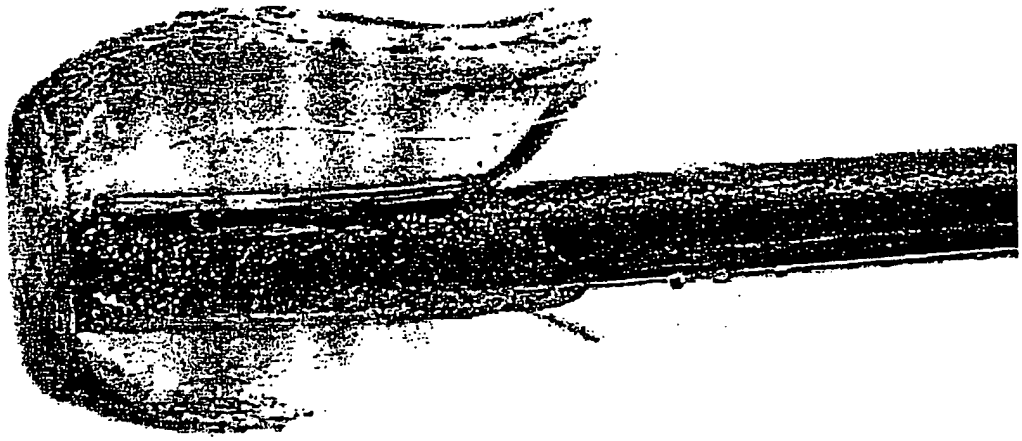


FIG. 3A

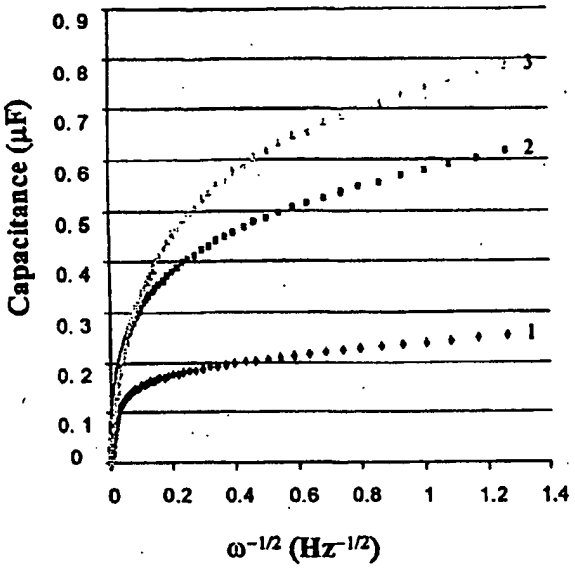


FIG. 3B

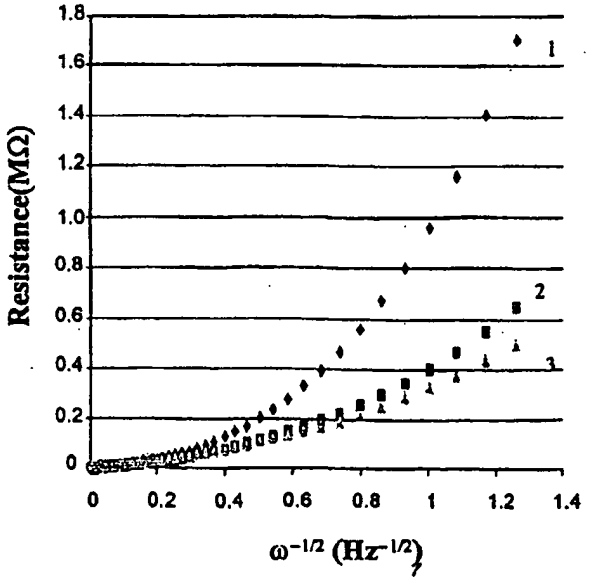


FIG. 4A

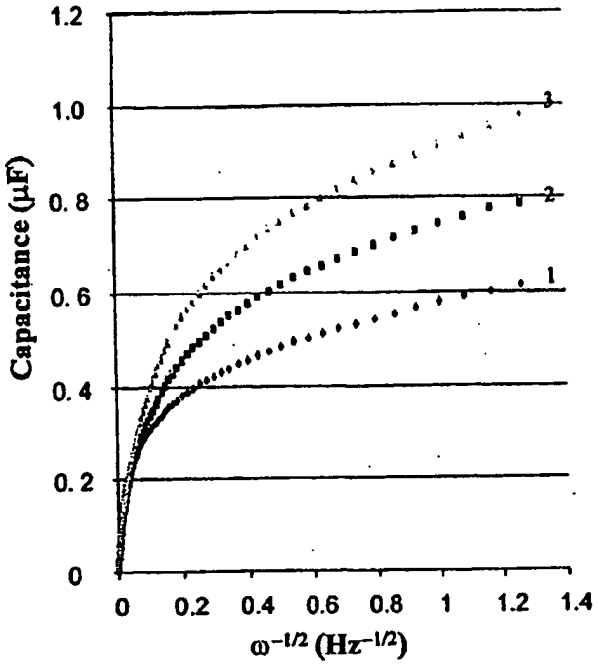


FIG. 4B